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Immunoblot Detection of Antibodies to Toscana Virus

Tino F. Schwarz, Sabine Gilch, Christof Pauli, and Gundula Jäger

Max von Pettenkofer Institute for Hygiene and Medical Microbiology, Ludwig Maximilians University, Munich, Germany

Sera from patients with sandfly fever caused by Toscana virus (TOSV) infection were tested by immunoblot for specific antibody response to TOSV derived from infected Vero-E6 cells. The 28 kDa TOSV nucleoprotein (N) was identified as the major immunodominant protein recognized by immunoblot. In sera of patients with acute TOSV infection, specific antibodies of the IgM, IgA, and IgG class were detected. Using sandfly fever virus, serotypes Sicilian (SFSV) and Naples (SFNV), as antigens for immunoblot, TOSV antibody-positive sera cross-reacted with the corresponding N proteins. These sera reacted for IgM and IgG by SFSV immunoblot, and for IgM by SFNV immunoblot. The diagnosis of sandfly fever may be confirmed by TOSV immunoblot.

KEY WORDS: Bunyaviridae, phlebovirus, sandfly fever virus, nucleoprotein, laboratory diagnosis

INTRODUCTION

Sandfly fever viruses (SFV) belong to the *Phlebovirus* genus of the Bunyaviridae. Like other members of the *Bunyaviridae,* sandfly fever viruses possess a tripartite negative-strand RNA genome with the large (L) segment coding for the virus polymerase, the medium (M) segment encoding the envelope glycoproteins G1 and G2, and the small (S) segment encoding the nucleoprotein (N) and the non-structural protein (NS_s). An ambisense coding strategy unique for the *Phlebovirus* and *Uukuvirus* genus was demonstrated for the S segment [Marriott et al., 1989; Giorgi et al., 1991]. The three SFV serotypes, Sicilian (SFSV), Naples (SFNV), and Toscana (TOSV), are antigenetically distinct but as demonstrated by complement fixation [Tesh et al., 1982] not by neutralization test [Eitrem et al., 1991a], TOSV and SFNV are more closely related. All three serotypes are present in the Mediterranean region, and are transmitted by *Phlebotomus* species. TOSV was isolated first in Italy from *Phlebotomus perniciosus* [Verani et al., 1980, 1984]. Recent studies in endogenous populations and case reports of infection in tourists indicate that TOSV is endemic in Italy [Calisher et al., 1987; Nicoletti et al., 1991; Schwarz et al., 1993, 1995a,b], Portugal [Ehrnst et al., 1985; Schwarz et al., 1995a], Spain [Eitrem et al., 1991b], and Cyprus [Eitrem et al., 1991a,b]. An antibody prevalence to TOSV of 0.8% was found recently during a seroepidemiological survey in Germany using an enzyme-immunoassay (EIA), indicating the significance of TOSV as a travel-related infection [Schwarz et al., 1995a].

Human disease, first described in Herzegovina [Pick, 1886], is now known as sandfly fever [pappataci fever; phlebotomus fever]. SFSV and SFNV generally cause a febrile illness with headache and sometimes mild neurological symptoms, whereas TOSV infection is characterized by fever, severe headaches, and aseptic meningitis complicated by transient cranial nerve palsy in some patients [Nicoletti et al., 1991; Schwarz et al., 1995b]. In TOSV infection, convalescence may be prolonged.

Following infection, viraemia is brief and lasts for up to 2 days. In patients with meningitis, TOSV has been isolated from cerebro-spinal fluid (CSF) in 14% of patients [Nicoletti et al., 1991]. Amplification of genomic TOSV RNA from serum and CSF by nested reverse-transcriptase polymerase chain reaction was been recently reported [Schwarz et al., 1995c]. At present, TOSV infection is diagnosed serologically by detecting specific antibodies (anti-TOSV IgM and IgG) by indirect immunofluorescence assay (IIFA), EIA, complement fixation, haemagglutination-inhibition-assay, and plaque reduction neutralization test (PRNT) [Tesh et al., 1982; Eitrem et al., 1991c; Nicoletti et al., 1991; Schwarz et al., 1995a].

This study describes the use of immunoblot for detecting specific TOSV antibodies using virus protein derived from native virus antigen.

MATERIALS AND METHODS

Clinical Specimens

A panel of sera and immunoglobulins with known TOSV antibody status was used for evaluating the immunoblot. The sera were obtained from German tourists with acute sandfly fever (n = 9) with anti-TOSV IgM and IgG who had been infected during visits to Italy. In addition, sera of individuals with past TOSV infection (n = 15), positive for anti-TOSV IgG but negative for

Address reprint requests to Dr. Tino F. Schwarz, M.D., Stiftung Juliusspital, Juliuspromenade 19, 97070 Würzburg, Germany.

anti-TOSV IgM, and sera negative for both markers (n = 30), were tested. The specificity of the immunoblot was determined by testing sera of patients with acute or past Epstein-Barr virus (EBV) (n = 10), cytomegalovirus (CMV) (n = 10), herpes simplex virus (HSV) (n = 10), varicella zoster virus (VZV) (n = 10), and mumps virus (n = 10) infection.

As antibody-positive control for TOSV, a human serum of a confirmed clinical case of TOSV infection was used, and as negative control, a serum negative for SFV markers was used. As antibody-positive controls for SFSV and SFNV, polyclonal mouse sera were used. Human SFSV and SFNV antibody-positive sera were not available for this study.

Virus Stocks

As reference TOSV strain for propagation in cell culture, TOSV ISS.Phl.3, isolated originally in Tuscany from *Ph. perniciosus* in 1971, was used [Verani et al., 1980]. In addition, reference strains of SFSV (SF Sicilian virus Oct-85, strain Sabin) and SFNV (SF Naples virus Oct-85, strain Sabin) were used to determine cross-reactivity of antibodies to the antigens of the three sandfly fever viruses.

Preparation of Virus Antigens

Virus antigen was prepared from TOSV-, SFSV-, and SFNV-infected Vero-E6 cells. Confluent monolayers of cultured cells in 175 mm^2 tissue culture flasks were infected with 1.5 ml of each virus suspension, and cultivated for 7 days in Eagle's MEM supplemented with 5% foetal calf serum. Supernatants and cells were collected, thawed, and frozen three times to release remaining virus from the cells, and centrifuged for 10 min at 4,000*g* to remove cell debris. A total volume of 70 ml supernatant was then centrifuged for 4 h at 75,000*g*. The supernatant was discarded and the pellet resuspended in 1 ml buffer (0.15 M NaCl, Tris-HCL (pH 7.4), EDTA (ph 8.0), sonicated for 10 min, aliquoted, and stored at −70°C until use.

An uninfected mock control was prepared in the same manner.

Enzyme-Immunoassay

All specimens were tested for anti-TOSV IgM and IgG by enzyme-immunoassay (EIA) using native virus antigen as described previously [Schwarz et al., 1995a]. Briefly, anti-TOSV IgM was measured using a "μ-capture assay" with a polyclonal mouse antibody to TOSV. Anti-TOSV IgG was determined by directly coating native virus antigens to the solid phase of flat-bottom microtitre plates [Schwarz et al., 1995a].

Immunoblot

The technique used is essentially similar to the method described previously for detecting antibodies to human immunodeficiency virus [Gürtler et al., 1987]. A 12.5% SDS-polyacrylamide gel was prepared for separating TOSV, SFSV, and SFNV suspensions, respectively. An equal volume of TOSV, SFSV, SFNV suspension, and electrophoresis buffer (0.065 M Tris, 9.2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromphenol blue) was heated at 100°C for 5 min, rapidly cooled in ice water and applied to the gel. A mock control was also prepared. After electrophoresis at 10 mA for 16 hr, proteins were transferred to nitrocellulose with blotting buffer (14.5 g/L glycine, 3 g/L tris base, 200 ml/L methanol) for 2 hr at 4°C, using charcoal plates as electrodes at 1 mA for 2 hr. The membrane was incubated in a 5% suspension of milk powder for 1 hr at 37°C. The membrane was cut in strips, and each strip was incubated with test serum at a dilution of 1:100 in phosphate buffered saline (PBS) overnight at room temperature. Strips were washed three times in washing buffer (50 mM Tris, pH 7.5, 5 mM Na_2EDTA, 150 mM NaCl, 0.25% gelatine, 0.5% Triton ×100, 0.1% SDS) for 45 min, and incubated with peroxidase-conjugated anti-human IgM, IgA and IgG rabbit immunoglobulin (dilution 1:500, 1:500 and 1:1,000 respectively, Dako, Hamburg) for 2 hr and washed 6 times with washing buffer for 45 min. Peroxidase-conjugated anti-mouse IgG (dilution 1:1,000) was used for SFSV- and SFNV-positive controls. Finally, the strips were developed with diaminobenzidine (0.02% diaminobenzidine tetrahydrochloride, 0.5 M Tris-HCl, pH 7.4, in 0.155 M NaCl, 0.09% H_2O_2).

Sera were preincubated with RF absorbens (Behringwerke, Marburg) for detecting specific IgM by immunoblot. One milliliter of the resuspended pellet prepared by ultracentrifugation was required for one gel (40 nitrocellulose strips).

RESULTS

Sera of Patients With Acute Sandfly Fever

All nine sera from patients with acute TOSV infection had anti-TOSV IgM and IgG by EIA. Anti-TOSV IgM, IgA, and IgG were detected by immunoblot. These TOSV antibody-positive sera reacted strongly with the 28 kDa band representing the TOSV N protein. Figure 1 shows the results of three of the nine sera of patients with acute TOSV infection. Using the mock control as antigen, a specific band was not detected (data not shown).

Cross-Reactivity With Other SFV Serotypes

To study cross-reactivity of TOSV antibody-positive sera with SFNV and SFSV, these two serotypes were used as antigen for the immunoblot. As shown in Figure 2, TOSV antibody-positive sera reacted with the SFNV N protein only for IgM, but not for IgA and IgG. Using SFSV as antigen, all TOSV antibody-positive sera reacted with SFSV N protein (Fig. 3). IgM and IgG were detected but not IgA.

Specificity

Of 50 sera of patients with acute EBV, VZV, HSV, CMV, and mumps infection, all of which were negative for anti-TOSV IgM and IgG by EIA, none was positive by TOSV immunoblot. These 50 sera were also antibody-negative using SFSV or SFNV as antigens for immunoblot.

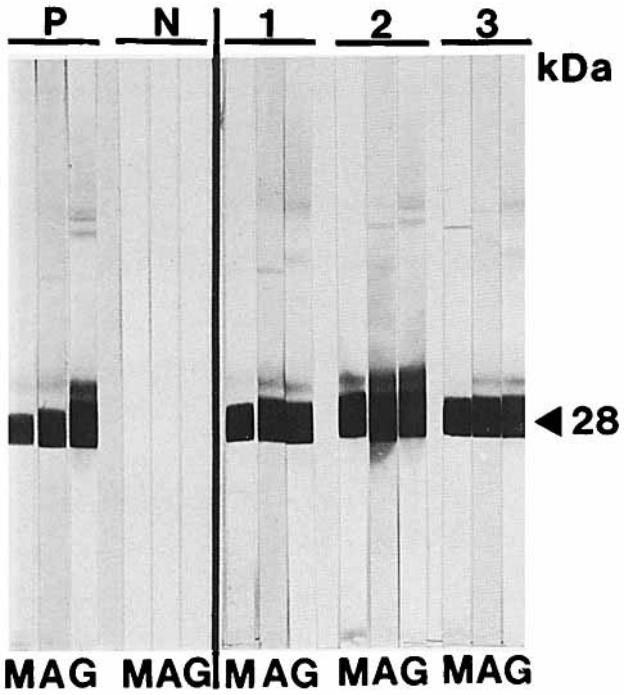

Fig. 1. Immunoblot of three sera (1–3) of patients with acute TOSV infection using TOSV as antigen. Anti-TOSV IgM (M), IgA (A), and IgG (G) reacted with the 28 kDa TOSV N protein. **P** is an antibody-positive control, and **N** is a negative control.

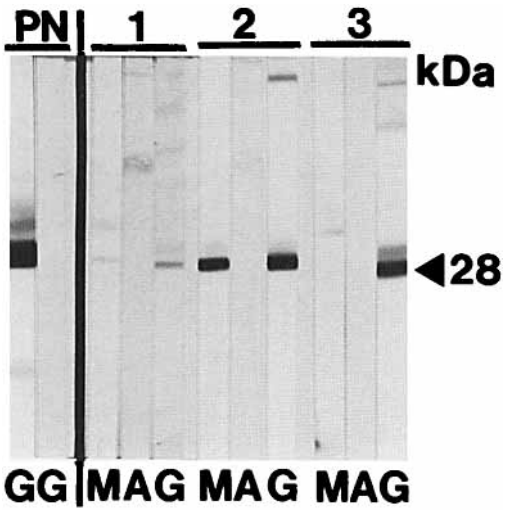

Fig. 2. Immunoblot of three TOSV-positive sera (same sera as shown in Fig. 1) using SFSV as antigen. **P** is a SFSV-antibody-positive mouse serum, and **N** is a negative control.

Sera of Individuals With Past Infection

All 15 sera of patients with past TOSV infection positive for anti-TOSV IgG by EIA reacted with the 28 kDa band by TOSV immunoblot. These sera were negative for anti-TOSV IgM and IgA but positive for anti-TOSV IgG. Using SFSV as antigen for immunoblot, these 15 anti-TOSV IgG-positive sera reacted strongly with the

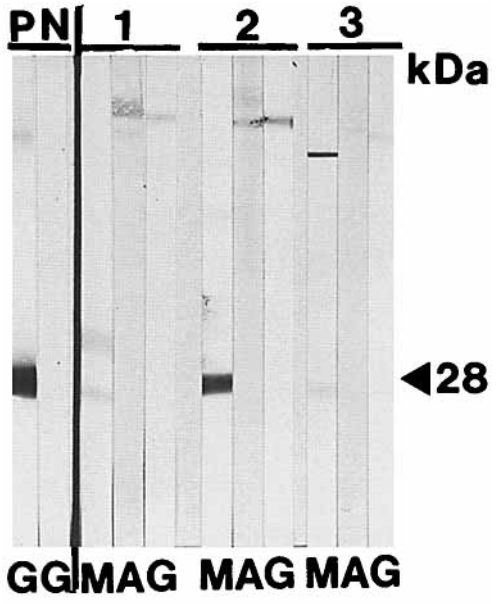

Fig. 3. Immunoblot of three TOSV-positive sera (same sera as shown in Fig. 1) using SFNV as antigen. **P** is a SFNV-antibody-positive mouse serum, and **N** is a negative control.

SFSV N protein for IgG. By SFNV immunoblot, anti-TOSV IgG-positive sera also reacted positive with the SFNV N protein for IgG, but produced only a weak band.

DISCUSSION

This study identified the 28 kDa N protein as the major virus antigen recognized by the humoral immune response in TOSV infection. In acute TOSV infection, specific IgM, IgA, and IgG are produced against the N protein. As in other infections with *Bunyaviridae,* only antibodies to the N protein but not to the glycoproteins G1 and G2 were detected. Specific TOSV antibodies were cross-reactive with N proteins of SFSV, and to a minor extent, with SFNV. Reverse reactivities could not be studied, since sera of patients with acute SFSV and SFSV infection were not available.

Studies based on complement fixation tests and immunofluorescence assays indicated that TOSV is antigenetically more related to SFNV than to SFSV [Eitrem et al., 1991a] but by immunoblot TOSV antibody-positive sera cross-reacted more strongly with SFSV than to SFNV. No cross-reactions between the different serotypes of SFV were reported using plaque-neutralization test [Eitrem et al., 1991a].

Immunoblots are now used for detecting antibodies to other *Bunyaviridae,* such as Hantaan (HTNV) virus, Puumala virus (PUUV), sin nombre virus (SNV), and Crimean-Congo haemorrhagic fever (C-CHFV). In hantavirus infection, sera reacted with a protein of 52 (PUUV) and 50 kDa (HTNV) representing the N proteins [Zöller et al., 1989]. As there is no ambisense coding strategy for the hantavirus S RNA, the gene product of the N gene is expected to have a higher molecular weight than the N protein of phleboviruses. It was shown that

recombinant N proteins of HTNV and PUUV expressed in *E. coli* can be used as immunodiagnostic antigens [Gött et al., 1991; Zöller et al., 1993]. Recently, it was demonstrated for PUUV that N protein is the dominant acute-phase antigen [Vapalahti et al., 1995]. A recombinant N protein of SNV was used for serological assays to detect antibodies in patients with hantavirus pulmonary syndrome [Feldmann et al., 1993]. A recent study demonstrated that sera of deer mice infected with sin nombre virus (SNV) reacted only with the N protein by immunoblot but not with the glycoproteins G1 and G2, and antibodies to SNV cross-reacted with recombinant N proteins of PUUV, HTNV, and Seoul virus [Yamada et *al., 1995*]. A recombinant N protein of C-CHFV expressed in *E. coli* was shown to be useful as antigen for immunoblot and EIA [Marriott et al., 1994].

To date, acute sandfly fever is diagnosed rarely in non-endemic countries. With increase in travel to endemic areas, especially the Mediterranean region, imported cases of sandfly fever are increasingly important in non-endemic countries. Sandfly fever may cause diagnostic problems since many physicians in non-endemic countries are unaware of the potential importation of this vector-borne virus disease. At present, laboratory diagnosis is hampered by the limited number of laboratories undertaking such tests. The use of TOSV immunoblot could provide more widespread availiability of laboratory diagnosis of sandfly fever.

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REFERENCES

Calisher CH, Weinberg AN, Muth DJ, Lazuick JS (1987): Toscana virus infection in United States citizen returning from Italy. The Lancet i:165–166.

Ehrnst A, Peters CJ, Niklasson B, Svedmyr A, Holmgren B (1985): Neurovirulent Toscana virus (a sandfly fever virus) in Swedish man after visit to Portugal. The Lancet i:1212–1213.

Eitrem R, Niklasson B, Weiland O (1991a): Sandfly fever among Swedish tourists. Scandinavian Journal of Infectious Diseases 23: 451–457.

Eitrem R, Vene S, Niklasson B (1991b): ELISA for detection of IgM and IgG antibodies to sandfly fever Sicilian virus. Research in Virology 142:387–394.

Eitrem R, Stylianou M, Niklasson B (1991c): High prevalence rates of antibody to three sandfly fever viruses (Sicilian, Naples and Toscana) among Cypriots. Epidemiology and Infection 107:685–691.

Feldmann H, Sanchez A, Morzunov S, Spiropoulou CF, Rollin PE, Ksiazek TG, Peters CJ, Nichol ST (1993): Utilization of autopsy RNA for the synthesis of the nucleocapsid antigen of a newly recognized virus associated with hantavirus pulmonary syndrome. Virus Research 30:351–367.

Giorgi C, Accardi L, Nicoletti L, Gro MC, Takehara K, Hilditch C, Morikawa S, Bishop DHL (1991): Sequences and coding strategies of the S RNAs of Toscana and Rift Valley fever viruses compared to those of Punto Toro, Sicilian sandfly fever, and uukuniemi viruses. Virology 180:738–753.

Gött P, Zöller L, Yang S, Stohwasser R, Bautz EKF, Darai G (1991): Antigenicity of hantavirus nucleocapsid proteins expressed in E. coli. Virus Research 19:1–16.

Gürtler LG, Everle J, Lorbeer J, Deinhardt F (1987): Sensitivity and specificity of commercial ELISA kits for screening anti-LAV/HTLV III. Journal of Virological Methods 15:11–23.

Marriott AC, Ward VK, Nuttall PA (1989): The S RNA segment of sandfly fever Sicilian virus: evidence for an ambisense genome. Virology 169:341–345.

Marriott AC, Polyzoni T, Antoniades A, Nuttall PA (1994): Detection of human antibodies to Crimean-Congo haemorrhagic fever virus using expressed viral nucleocapsid protein. Journal of General Virology 75:2157–2161.

Nicoletti L, Verani P, Caciolli S, Ciufolini MG, Renzi A, Bartolozzi D, Paci P, Leoncini F, Padovani P, Traini E, Baldereschi M, Balducci M (1991): Central nervous system involvement during infection by phlebovirus Toscana of residents in natural foci in central Italy (1977–1988). American Journal of Tropical Medicine and Hygiene 45:429–434.

Pick A (1886): Zur Pathologie und Therapie einer eigenthümlichen endemischen Krankheitsform. Wiener Medizinische Wochenschrift 36:1141–1145.

Schwarz TF, Gilch S, Jäger G (1993): Travel-related Toscana virus infection. The Lancet 342:803–804.

Schwarz TF, Jäger G, Gilch S, Pauli C (1995a): Serosurvey and laboratory diagnosis of imported sandfly fever virus, serotype Toscana, infection in Germany. Epidemiology and Infection 114:501–510.

Schwarz TF, Gilch S, Jäger G (1995b): Aseptic meningitis caused by sandfly fever virus, serotype Toscana. Clin Infect Dis 21:669–671.

Schwarz TF, Jäger G, Gilch S, Nitschko H (1995c): Nested RT-PCR for detection of sandfly fever virus, serotype Toscana, in clinical specimens, with confirmation by nucleotide sequence analysis. Research in Virology 146:355–362.

Tesh RB, Peters CJ, Meegan JM (1982): Studies on the antigenetic relationship among phleboviruses. American Journal of Tropical Medicine and Hygiene 31:149–155.

Vapalahti O, Kallio-Kokko H, Närvänen A, Julkunen I, Lundkvist A, Plyusnin A, Lehväslaiho H, Brummer-Korvenkontio M, Vaheri A, Lankinen H (1995): Human B-cell epitopes of Puumala virus nucleocapsid protein, the major antigen in early serological response. Journal of Medical Virology 46:293–303.

Verani P, Lopes MC, Nicoletti L, Balducci M (1980): Studies on phlebotomus-transmitted viruses in Italy: I. Isolation and characterization of a sandfly fever Naples-like virus. Zbl Bakt (suppl 9):195–201.

Verani P, Nicoletti L, Ciufolini MG (1984): Antigenetic and biological characterization of Toscana virus, a new phlebotomus fever group virus in Italy. Acta Virol 28:39–47.

Yamada T, Hjelle B, Lanzi R, Morris C, Anderson B, Jenison S (1995): Antibody responses to Four Corners hantavirus infections in the deer mouse (Peromyscus maniculatus): identification of an immunodominant region of the viral nucleocapsid protein. Journal of Virology 69:1939–1943.

Zöller L, Scholz J, Stohwasser R, Giebel LB, Sethi KK, Bautz EKF, Darai G (1989): Immunoblot analysis of the serological response in hantavirus infections. Journal of Medical Virology 27:231–237.

Zöller L, Yang S, Gött P, Bautz EKF, Darai G (1993): Use of recombinant nucleocapsid proteins of the Hantaan and nephropathia epidemica serotypes of hantaviruses as immunodiagnostic antigens. Journal of Medical Virology 39:200–207.